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among infants with bronchiolitis: A multiomic analysis.](#)

American Journal of Respiratory and Critical Care Medicine 2017, 196(7),
pp. 882-891.

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This is the authors' accepted manuscript of an article that has been published in its final definitive form by the American Thoracic Society, 2017.

DOI link to article:

<https://doi.org/10.1164/rccm.201701-0071OC>

Date deposited:

05/01/2018

**Associations of nasopharyngeal metabolome and microbiome with severity among
infants with bronchiolitis: A multi-omic analysis**

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Author Contributions: Conception and design: C.J.S, J.M.M, C.A.C, and K.H. Acquisition of data: C.J.S, J.M.M, N.J.A, J.F.P, C.A.C, and K.H. Analysis and interpretation of data: C.J.S, J.M.M, M.C.W, and K.H. Drafting or revising the article: C.J.S, J.M.M, C.A.C, and K.H. Acquisition of data: C.J.S, J.M.M, N.J.A, J.F.P, C.A.C, and K.H. Final approval of the manuscript: C.J.S, J.M.M, M.C.W, N.J.A, J.F.P, C.A.C, and K.H.

Funding: This work was supported by the grants U01 AI-087881, R01 AI-114552, R01 AI-108588, R21 HL-129909, R01 AI-127507, and UG3 OD-023253 from the National Institutes of Health (Bethesda, MD). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Running head: Metabolome and microbiome with bronchiolitis

Descriptor: Microbiology and pulmonary infections: 10.9 Pathogen/Host Cell Interactions

Word count: 3497

At a Glance Commentary

Scientific Knowledge on the Subject: Bronchiolitis disease severity varies significantly among infants, which cannot be explained by currently known risk factors. Emerging evidence indicates that bronchiolitis pathobiology involves a complex interplay among viruses, airway microbiome, and host immunity. However, a detailed assessment of the airway metabolome and bacterial metagenome (microbiome) has not been performed.

What This Study Adds to the Field: In this multicenter cohort study, infants with more-severe bronchiolitis – defined by the use of positive pressure ventilation – had significantly altered nasopharyngeal airway metabolome profiles compared to those with less-severe bronchiolitis. Increased sphingolipid metabolism was correlated with higher relative abundance of *Streptococcus pneumonia* and was associated with higher bronchiolitis severity. Our data highlight microbe-host interactions in the airway of infants, which may provide a novel therapeutic strategy to treat infants with bronchiolitis.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

ABSTRACT

Rationale: Bronchiolitis is the most common lower respiratory infection in infants; however, it remains unclear which infants with bronchiolitis will develop severe illness. In addition, while emerging evidence indicates associations of the upper-airway microbiome with bronchiolitis severity, little is known about the mechanisms linking airway microbes and host response to disease severity.

Objectives: To determine the relations among the nasopharyngeal airway metabolome profiles, microbiome profiles, and severity in infants with bronchiolitis.

Methods: We conducted a multicenter prospective cohort study of infants (age <1 year) hospitalized with bronchiolitis. By applying metabolomic and metagenomic (16S rRNA gene and whole genome shotgun sequencing) approaches to 144 nasopharyngeal airway samples collected within 24 hours of hospitalization, we determined metabolome and microbiome profiles and their association with higher severity, defined by the use of positive pressure ventilation (PPV) – i.e., continuous positive airway pressure and/or intubation.

Measurements and Main Results: Nasopharyngeal airway metabolome profiles significantly differed by bronchiolitis severity ($P<0.001$). Among 254 metabolites identified, a panel of 25 metabolites showed high sensitivity (84%) and specificity (86%) in predicting the use of PPV. The intensity of these metabolites was correlated with relative abundance of *Streptococcus pneumoniae*. In the pathway analysis, sphingolipid metabolism was the most significantly enriched sub-pathway in infants with PPV use compared to those without ($P<0.001$). Enrichment of sphingolipid metabolites was positively correlated with the relative abundance of *S. pneumoniae*.

Conclusions: While further validation is needed, our multi-omic analyses demonstrate the potential of metabolomics to predict bronchiolitis severity and better understand microbial-host interaction.

INTRODUCTION

Bronchiolitis, an acute lower respiratory viral infection, is an important public health problem in the U.S and worldwide. Indeed, bronchiolitis is the leading cause of infant hospitalizations in the US, accounting for 18% of infant hospitalizations (approximately 130,000 hospitalizations annually) (1, 2). While approximately 40% of children develop clinical bronchiolitis in the first two years of life (3), its severity ranges from a minor nuisance to fatal (4). Studies have reported several clinical risk factors for higher severity (e.g., prematurity, comorbidity); however, it remains unclear which children with bronchiolitis will develop severe illness (5), such as bronchiolitis requiring positive pressure ventilation (PPV) support.

Emerging evidence indicates that bronchiolitis pathobiology involves a complex interplay among viruses, airway microbiome, and host immunity (6, 7). For instance, dominance of *Streptococcus* or *Haemophilus* in the upper airway has been associated with higher disease severity (7–9). Yet, little is known about molecular mediators of this interplay. Metabolomics, the systematic analysis of functional small-molecules, present pathobiological profiles that encompass microbial and host interactions (10–12). For a number of complex infectious and inflammatory diseases such as sepsis (13), asthma (14, 15), and cystic fibrosis (CF) (16, 17), metabolomic approaches have identified new biomarkers and novel pathobiological pathways. However, to date, no study has applied a metabolomics approach to infants with bronchiolitis.

To address these knowledge gaps, we used nasopharyngeal airway samples from a multicenter prospective study of infants hospitalized for bronchiolitis to profile the airway metabolome and microbiome, and to determine their relation to disease severity with focusing on the use of PPV. The current study also utilized 16S rRNA gene sequence data published previously (6, 8, 18).

MATERIALS AND METHODS

Study design, setting, and participants

As part of a 17-center, prospective cohort study of 1,016 infants (age <1 year) hospitalized for bronchiolitis, based on *a priori* defined study aim, the current investigation analyzed the data of 144 infants with nasopharyngeal metabolomic and microbiome testing and determined their relation to disease severity. This cohort, called the 35th Multicenter Airway Research Collaboration (MARC-35) (6), was coordinated by the Emergency Medicine Network (EMNet) – a research collaboration comprised of 245 hospitals. The study design, setting, participants, and methods of data collection have been reported previously (6, 8). In brief, MARC-35 site investigators at 17 sites across 14 U.S. states enrolled infants hospitalized with an attending physician diagnosis of bronchiolitis during three consecutive bronchiolitis seasons from November 1 to April 30 (2011-2014). Bronchiolitis was defined by the American Academy of Pediatrics guidelines – acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions (19). We excluded infants who were transferred to a participating hospital >24 hours after the original hospitalization, those who were consented >24 hours after hospitalization, or those with known heart-lung disease, immunodeficiency, or gestational age <32 weeks. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Nasopharyngeal airway sample collection

Nasopharyngeal samples were collected by trained site investigators using the same standardized protocol utilized in a previous cohort study of children with bronchiolitis (20, 21). Briefly, 1 mL of normal saline was instilled into one naris, and mucus was removed by

means of an 8 French suction catheter. The procedure was performed once on each nostril and following sample collection from both nares, 2 mL of normal saline was suctioned through the catheter to clear the tubing and ensure that a standard volume of aspirate was obtained. All sites used the same collection equipment (Medline Industries, Mundelein, IL) and collected the samples within 24 hours of hospitalization. The nasopharyngeal sample was immediately placed on ice and then stored at -80°C until the samples were tested for nasopharyngeal airway metabolome and microbiome.

Metabolome testing

Metabolome testing used 125 μL of nasopharyngeal sample and was performed by Metabolon (Durham, NC). All samples were blinded to Metabolon and processed in a random order. Metabolome profiling used ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Details of the sample preparation, metabolome profiling, identification of compounds, and quality control may be found in the Online Supplement (Supplementary Methods)(22).

Microbiome testing

Microbial DNA was extracted from the nasopharyngeal samples using MO BIO PowerSoil DNA Isolation Kit (Mo Bio Laboratories; Carlsbad, CA) as described previously (8). The 16S rRNA gene sequencing data were generated in earlier work (8). The sequencing methods were adapted from those developed for the NIH-Human Microbiome Project (23, 24). In brief, the 16S rDNA V4 region was amplified by PCR using barcoded Illumina adapter-containing primers 515F and 806R (25) and sequenced on the MiSeq platform (Illumina; San Diego, CA) using the 2x250 bp paired-end protocol yielding pair-end reads that overlap almost completely. Demultiplexing of reads was performed using USEARCH

v7.0.1090 (26) and quality filtering was performed as previously described (8). Resulting OTU tables were rarified to 1,500 reads per sample.

To examine the encoded metabolic potential of nasopharyngeal airway microbiome, the current study also applied a metagenomic whole genome shotgun (WGS) sequencing to 70 nasopharyngeal samples. Individual libraries constructed from each sample were sequenced using the 2x100 bp paired-end read protocol on the HiSeq platform (Illumina). The process of quality filtering, trimming and demultiplexing was performed using a pipeline developed at the Baylor College of Medicine that employs a number of publicly available tools such as Casava v1.8.3 (Illumina) for the generation of fastqs, Trim Galore and cutadapt for adapter and quality trimming, and PRINSEQ for sample demultiplexing. Additionally, Bowtie2 v2.2.1 was used to map reads to custom databases for bacteria, viruses, human, and vectors and remove non-bacterial reads from the dataset. For bacterial reads, the highest identity match was chosen. If there were multiple top hits, the lowest common ancestor was determined, but these reads do not contribute to the analysis. Reads whose genomic coordinates overlapped with known KEGG orthologs (K numbers) were tabulated. Coding sequences from references genomes that have not been specifically annotated by KEGG were aligned to all known KEGG orthologs. Any coding sequence that had >70% identity and >70% query coverage to a known KEGG ortholog was assigned to that KEGG ortholog. This in effect created links between new genomes and the KEGG database. KEGG modules (M numbers) were calculated step-wise and determined to be complete if 65% of the reaction steps were present per detected species and for the metagenome as a whole. Pathways were constructed for each taxa and metagenome by calculating the minimum set through MinPath (27) resulting from the gene orthologs present.

Details of the microbial DNA extraction, 16S rRNA gene sequencing, and metagenomic WGS sequencing may be found in the Online Supplement. Microbiome data

have been deposited in the NCBI BioProject (ID PRJNA356075).

Statistical analyses

The primary outcome measure was the use of PPV support – defined as the use of continuous positive airway pressure and/or intubation – at any time during the index hospitalization (20, 28). The analysis followed a workflow to determine the relations among the nasopharyngeal airway metabolome profiles, microbiome profiles, and use of PPV (**Figure E1**). Full analytical methodology is described in the Online Supplement. First, to examine the differences in nasopharyngeal metabolomic profile between infants with PPV use and those without, orthogonal partial least squares-discriminatory analysis (OPLS-DA) was performed (29) and validated by permutation testing (2000 permutations). Individual metabolites that significantly differ by PPV use were determined using two-tailed Welch's t-test in MetaboAnalyst 3.0 (30) and multivariable linear regression in R version 3.3 (31). The models adjusted for potential confounders (age, sex, history of prematurity, history of antibiotic use prior to the enrollment, use of systemic corticosteroids during the pre-hospitalization visit, virus, and hospital site). P-values were adjusted for multiple comparisons with the false discovery rate (FDR) algorithm (32). To determine the predictivity of individual metabolites as a marker for PPV use, receiver operating characteristic (ROC) curves were generated by linear support vector machine (SVM) classification with Monte-Carlo cross validation. In each Monte-Carlo cross validation, two-thirds of the samples were used to examine the feature importance based on weighted coefficients, and the important features (metabolites) were used to build classification models which were validated using the one-third of samples left out. Additionally, to examine pathways that are differentially enriched in infants who underwent PPV, pathway enrichment analysis was performed. Next, MixOmics (33) was implemented in R to determine the correlation between bacterial taxa and

the intensity of metabolites of interest with the use of sparse partial least squares regression in canonical mode (34). Furthermore, in order to determine the severity-related differences in metabolic potential of the nasopharyngeal microbiome overall and bacterial genera, two-tailed Welch's t-test was used to compare the metagenomic KEGG orthologs (KOs). Lastly, to determine biologically feasible correlations between the metagenomic KOs and the measured metabolites of likely bacterial origin, the Model-based Integration of Metabolite Observations and Species Abundances (MIMOSA) method was used (35).

RESULTS

Study population and analysis workflow

As part of 17-center prospective cohort study of 1,016 infants hospitalized for bronchiolitis, the current investigation analyzed 144 infants with sufficient amount of nasopharyngeal airway sample for metabolome and microbiome testing. The analytic and nonanalytic cohorts had no significant differences in most patient characteristics ($P>0.05$; **Table E1**), except the analytic cohort had a relatively higher proportion of rhinovirus infection (28% vs. 20%; $P=0.04$). Of 144 infants in the analytic cohort, the median age was 3 months (IQR, 1–6 months) and 25 (17%) underwent PPV during their hospitalization. Infants who underwent PPV were younger and more likely to have respiratory syncytial virus (RSV) infection compared to those without (both $P<0.05$; **Table 1**).

Metabolomics analysis of individual metabolites showed nasopharyngeal metabolomic profiles significantly differed by PPV use among infants with bronchiolitis

Metabolomic analysis detected a total of 254 metabolites, from 62 sub-pathways, contained within 8 super-pathways, in the nasopharyngeal airway of infants with

bronchiolitis. The metabolomic profiles (i.e., normalized intensity of individual metabolites, irrespective of sub- and super-pathway) clustered distinctly between infants with PPV use and those without PPV use in OPLS-DA (**Figure E2**). The significant difference was validated using permutation testing ($P < 0.001$). The individual metabolites that discriminated the infants with PPV use from those without included all 20 detected metabolites from the sphingolipid metabolism sub-pathway (**Figure 1**). By contrast, 5-oxoproline, trans-urocanate, 13-HODE-9-HODE, and 15-HETE were negatively associated with the risk of PPV use.

The intensity of these individual metabolites in the infants' nasopharyngeal airway differed by PPV use, with no single metabolite ubiquitously increased among all infants with PPV use and decreased among all infants without PPV use, or *vice versa*. Thus, to determine the predictivity of individual metabolites as a marker for higher severity (i.e., use of PPV) at the point of hospitalization, we employed multivariate ROC curve analysis (**Figure 2A**). The ROC curve for the top 5, 10, 15, 25, 50, and 100 metabolites showed the area-under-the-curve improved with a larger number of metabolites. Model 4 (25 metabolites) and model 6 (100 metabolites) achieved the highest accuracy with both having a sensitivity of 84% and specificity of 86% (**Table E2**). Given the equivalent accuracy, model 4 was selected as most appropriate. Of these 25 metabolites, 12 predicted a higher risk of PPV use and 13 predicted a lower risk (**Figure 2B**). The model performance was validated using permutation testing ($P < 0.001$ with 1000 random permutations).

Metabolomics analysis of metabolic pathways showed sphingolipid metabolism is significantly enriched among infants who underwent PPV

Previous analysis of individual metabolites, irrespective of the sub-pathway, demonstrated that the metabolomic profiles differ by PPV use and that 25 metabolites accurately predicted PPV use. While these 25 individual metabolites were the most predictive,

a total of 103 nasopharyngeal airway metabolites (from 6 super-pathways) significantly differed between infants with PPV use and those without (**Figure E3**). Moving beyond the analysis of individual metabolites, we next investigated metabolic pathway enrichment to determine which metabolic sub-pathways were significantly associated with risks of PPV use.

In accordance with the OPLS-DA (**Figure 1**), pathway enrichment analysis demonstrated that the sphingolipid metabolism sub-pathway (super-pathway: lipid) was the most significantly associated with risks of PPV use ($P<0.001$; **Figure E4**). Indeed, all 20 detected metabolites from this pathway were significantly more abundant in infants with PPV use compared to those without. Even after adjusting for potential confounders, 19 out of the 20 metabolites remained significant (all $P<0.05$; **Figure 3** and **Table E3**). To address the possibility of reverse causation (i.e., the use of PPV enhanced sphingolipid metabolism), we repeated the analysis with the use of another severity outcome – hospital length-of-stay ≥ 3 days (8). In this sensitivity analysis, the association between up-regulated sphingolipid metabolism and higher disease severity persisted, with 17 out of 20 sphingolipid metabolites remaining significant with adjustment for potential confounders (all $P<0.05$; **Table E3**).

Although many metabolites do not currently have corresponding KEGG compound identifiers, 5 of the 20 significant sphingolipid metabolites had corresponding KEGG compound numbers and mapped throughout the KEGG sphingolipid metabolism pathway (**Figure E5**). Other sub-pathways with significant enrichment included the phosphatidylethanolamine ($P<0.001$), plasmalogen ($P<0.001$), and phosphatidylcholine ($P<0.001$) from the lipid super-pathway, and the glycine, serine, and threonine metabolism ($P=0.02$) sub-pathway from the amino acid super-pathway (**Figures E3** and **E4**).

Microbiome and metabolomic analysis showed *Streptococcus* relative abundance positively correlates with intensities of metabolites that predict risks of PPV use and with enrichment of sphingolipid metabolism pathway

Taxonomic data generated by 16S rRNA gene sequencing and metagenomic WGS were comparable, showing *Haemophilus*, *Moraxella*, and *Streptococcus* genera dominated nasopharyngeal airway of infants with bronchiolitis (**Figure E6A and B**). The relative abundance of these genera was also comparable between sequencing approaches (**Figure E7**). The metagenomic sequencing (n=70; **Table E4**) permitted species-level identification and further demonstrated that *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* specifically dominated the nasopharyngeal airway (**Figure E6C**).

To determine the potential microbe-host interaction in the airway of infants with bronchiolitis, we examined the correlations between the relative abundance of the 3 dominant bacterial genera (*Streptococcus*, *Moraxella*, and *Haemophilus*) with the relative intensity of selected metabolites that were identified in the previous analyses (metabolites in model 4 and sphingolipid metabolism). The relative abundance of *Streptococcus* was positively correlated with the majority (11/12) of metabolites predicting high risks of PPV use from model 4 (**Figure 4A**). By contrast, the relative abundance of *Moraxella* and *Haemophilus* was generally negatively correlated with relative intensity of the same metabolites. These correlations were comparable in the sphingolipid metabolism pathway analysis, in which the relative abundance of *Streptococcus* was also positively correlated with the relative intensity of all metabolites from the sphingolipid metabolism pathway (**Figure 4B**).

Bacterial gene orthologs are distinct between infants with PPV use and those without

Lastly, the metagenome was analyzed to investigate potential differences in the metabolic potential of the nasopharyngeal microbiome according to use of PPV.

Metagenomic sequencing identified a total of 285 KOs that were differentially enriched between infants with PPV and those without (173 KOs vs 112 KOs, respectively; all $P < 0.05$; **Table E5**). KOs significantly enriched in infants with PPV use were primarily from *Streptococcus* sp. (33 KOs), *Rothia* sp. (33 KOs) and *Klebsiella* sp. (27 KOs). Additionally, 6 KOs from *Haemophilus* sp. were also significantly enriched among infants with PPV use. Conversely, all 112 KOs significantly enriched in infants without PPV use were from *Moraxella catarrhalis*.

At the individual metabolite level, MIMOSA analysis demonstrated the metagenomic KOs were significantly correlated with 15 measured metabolites, primarily amino acids (**Table E6**). Notably, the abundance of seryl-tRNA synthetase (KO1875) from *Streptococcus pneumoniae* was correlated with the intensity of serine (substrate of sphingosine). However, there was no significant correlation between the abundance of metagenomic KOs and the intensity of the metabolites that were significantly altered by PPV use (e.g. metabolites from sphingolipid metabolism), suggesting that the PPV-related measured metabolites (and pathways) are likely host derived.

DISCUSSION

In a multicenter prospective cohort of infants hospitalized for bronchiolitis, we used three complementary methodologies to characterize the nasopharyngeal airway metabolome, microbiome composition, and metagenome in samples collected at hospitalization. This is the first multi-omic analysis integrating these distinct methodologies to determine the association of airway metabolome and microbiome with severity of illness in infants with bronchiolitis. We found that the nasopharyngeal airway metabolome profiles significantly differed by PPV use. A panel of 25 selected metabolites provided high sensitivity (84%) and specificity (86%)

in predicting the use of PPV. Strikingly, all detected metabolites from the sphingolipid metabolism sub-pathway were discriminatory for PPV use by OPLS-DA, and sphingolipid metabolism was the most significantly enriched sub-pathway among infants with PPV use, with 19 out of 20 metabolites within this pathway significant even after adjusting for potential confounders. These findings were further validated using length of hospitalization as another marker for severity. The data also demonstrated that the association between higher abundances of *Streptococcus* and enriched sphingolipid metabolites, suggesting that the alterations of these metabolites are the result of a combination of microbial and metabolic activity.

Sphingolipids are not only integral components of the eukaryotic cell membrane, but also have molecular signaling functions with important roles in inflammation, immune response to infections, stress response, cell proliferation, and apoptosis (36–38). While the current study is the first to apply metabolomics to infant nasopharyngeal samples, metabolites associated with sphingolipid metabolism have been reported to increase in a range of existing studies investigating pulmonary disorders in childhood and adulthood (36, 37). For example, sphingolipids were primarily significantly increased in sputum from adults with CF compared to healthy controls (17). Additionally, sphingolipids directly promoted airway inflammation and were increased in the bronchoalveolar lavage fluid in patients with asthma compared with healthy controls (39, 40). Furthermore, multi-omic analysis of plasma in children with asthma also demonstrated altered metabolic functioning, primarily due to increased sphingolipid metabolism (15).

In a different line of inquiry, novel therapeutic agents have also targeted sphingolipids. It has been shown in an asthma murine model that inhalation of a sphingosine kinase inhibitor reduced inflammation and airway hyperresponsiveness, and improved immune responses (41). In a more recent murine study, inhibition of sphingosine kinase

suppressed pro-inflammatory NF- κ B and reduced airway inflammation (42). While these aforementioned studies do not directly investigate bronchiolitis, they link sphingolipid metabolism to inflammatory-mediated pathogenesis of airway diseases, and thereby support a potential role of sphingolipids in the pathobiology of bronchiolitis.

Amplicon sequencing of upper airway samples from children with respiratory infection has demonstrated an association between microbial dominance in the airway and illness severity. *Moraxella* has been associated with decreased severity of illness in infants hospitalized with bronchiolitis (8), while *Streptococcus* or *Haemophilus* have been associated with increased severity (7, 9, 43) and reduced microbiome stability (44). Consistent with these studies, our multi-omic analysis demonstrated that the abundance of *Moraxella* was generally negatively correlated with the intensity of metabolites associated with PPV use, and *M. catarrhalis* contributed to all metagenomes significantly enriched among infants without PPV use. By contrast, the relative abundance of *Streptococcus* was positively correlated with the intensity of PPV-associated metabolites. These bacteria-metabolite correlations persisted for both the individual metabolites (model 4) and the sphingolipid metabolism sub-pathway.

Our multi-omic analysis brings together microbiome and metabolome data and yielded findings that are concordant with recent studies exploring respiratory infections. In adults with CF, *Streptococcus* abundance in sputum was correlated with the relative intensity of metabolites involved in sphingolipid metabolism, including ceramide (18:2/16:0) and sphingomyelin (16:1/16:0) (16). A separate study investigating adults with *S. pneumonia* and *H. influenza* pneumonia and controls showed that plasma sphingolipids were primarily responsible for discrimination between patients with pneumonia and controls (45). Interestingly, our data also demonstrated that the metagenome-derived function of nasopharyngeal microbiome is not directly correlated with the intensity of metabolites that are associated with PPV use. This suggests that the significantly discriminant metabolites and

pathways (e.g., sphingolipid pathway) are unlikely to be microbiome-derived, but rather produced by the host. While further work is necessary to confirm this, most bacteria do not produce sphingolipids, which is especially true for aerobic bacteria, such as those found in the airways (46). Nevertheless, it is notable that the metagenomic data demonstrated *S. pneumoniae* to generate serine, a substrate of sphingosine – the fundamental building block of all sphingolipids. Thus, while the sphingolipid metabolites are host derived, exogenous serine generated by *S. pneumoniae* may contribute to the significantly increased sphingolipid metabolism in the airway of infants with bronchiolitis.

The nature of the observed airway microbiome composition-metabolome-severity relations warrants clarification. It is possible that the airway microbiome contributed to higher bronchiolitis severity by modulating host cellular function and metabolism (e.g., sphingolipid metabolism). Alternatively, following a change in host metabolism certain species (e.g., *S. pneumoniae*) were able to proliferate (47–49). Additionally, reverse causation is possible – i.e., more severe bronchiolitis not only led to an overgrowth of specific bacteria in the airway niche, but also altered host cellular metabolism. We also recognize that these mechanisms are not mutually exclusive. Our data should facilitate further research to elucidate the underlying mechanisms linking the microbes, host immune response, and altered metabolism in the airway to bronchiolitis pathogenesis.

Potential limitations

The current study has several potential limitations. First, nasopharyngeal samples were used due to technical and ethical restrictions in sampling lower airways from young infants. However, studies have shown that upper airway sampling provides reliable representation of the lung microbiome (50, 51) and gene expression profiles (as a proxy for cellular function) in children (52). Second, this study was not able to directly prove causality,

but depletion of sphingolipid metabolism prevents disease onset in murine models of inflammatory mediated respiratory diseases, supporting our data for the role of this sub-pathway in bronchiolitis severity (41, 42). Third, while the current study demonstrated the findings to be robust by performing analytical validation, external validation would be necessary to confirm the observations. Fourth, the inferences using 16S rRNA gene sequencing data were potentially limited because of their compositionality and may have led to false positives in correlation and statistical analyses (53, 54). Fifth, while the intensity of sphingolipids was significantly associated with risks of PPV use independently from age, history of antibiotic use, and other adjusted covariates, it is possible that some of observations might have been confounded by age. Additionally, our binary adjustment of antibiotic use assumes any exposure to antibiotics to be comparable, regardless of type and duration. Potential effects of antibiotic type and time course were not accounted for in our models. Sixth, we did not have information from a “control” group, such as infants with non-infectious disease. Yet, the objective of the current study was not to examine the role of metabolome and microbiome on the development of bronchiolitis but to determine their relationships with disease severity. Last, longitudinal studies would advance understanding of temporal changes in bacterial taxa and metabolites prior to bronchiolitis onset. Nonetheless, investigations into disease severity among infants hospitalized with bronchiolitis remain an important research focus given the high incidence (the leading cause of infant hospitalization).

Conclusions

In summary, this multi-omics analysis of nasopharyngeal samples collected prospectively from a multicenter cohort of infants hospitalized for bronchiolitis demonstrated that the metabolomic profiles differ by use of PPV (clinically important marker of disease

severity). We also found that, among 254 metabolites identified, a panel of 25 metabolites showed high sensitivity and specificity in discerning the use of PPV. Although external validation of the present findings is required, the ability to predict disease severity, specifically the need for respiratory support, has important implications for the clinical management of infants with bronchiolitis. Furthermore, our data demonstrated that the sphingolipid metabolism pathway was the most significantly enriched sub-pathway among infants who underwent PPV and that its enrichment was positively correlated with the abundance of *Streptococcus*. Emerging data suggest several important roles that sphingolipid metabolism may play in airway disease pathogenesis. Our findings support further experimental investigations to define the function of sphingolipids in acute respiratory infections, which may provide a novel therapeutic strategy to treat infants with severe bronchiolitis.

ACKNOWLEDGMENTS

The authors thank Ashley Sullivan, Courtney Tierney, and Janice Espinola, at the EMNet Coordinating Center (Boston, MA), and all of the site investigators and study staff for their valuable contributions to the MARC-35 study. We also thank Dr. Alkis Togias at NIH (Bethesda, MD) for helpful comments about the study results. Lastly, we thank the participating families for making all of this possible.

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TABLE**Table 1. Characteristics of 144 infants hospitalized for bronchiolitis by use of positive pressure ventilation**

Characteristics	Use of positive pressure ventilation (n = 25)	No positive pressure ventilation use (n = 119)	P Value
Age (mo), median (IQR)	1.4 (1.0-3.0)	3.0 (1.4-5.7)	0.02
Male sex	14 (56%)	74 (62%)	0.56
Race/ethnicity			0.21
Non-Hispanic white	15 (60%)	55 (46%)	
Non-Hispanic black	2 (8%)	22 (18%)	
Hispanic	5 (20%)	36 (30%)	
Other	3 (12%)	6 (5%)	
Maternal smoking during pregnancy	2 (8%)	10 (8%)	0.98
Prematurity (32-37 weeks)	4 (16%)	27 (23%)	0.46
C-section delivery	9 (36%)	44 (37%)	0.78
Postnatal smoke exposure at home	0 (0%)	16 (13%)	0.052
Number of siblings, median (IQR)	1 (0-5)	1 (0-5)	0.45
History of antibiotic use prior to enrollment	6 (24%)	41 (34%)	0.3
History of corticosteroid use prior to enrollment	3 (12%)	19 (16%)	0.61
Virology			
RSV infection	23 (92%)	85 (71%)	0.03
Rhinovirus infection	3 (12%)	37 (31%)	0.053
Neither RSV nor rhinovirus*	2 (8%)	3 (3%)	0.21
Positive pressure ventilation use			
Non-invasive positive pressure ventilation	14 (56%)	0 (0%)	—
Invasive positive pressure ventilation	18 (72%)	0 (0%)	—
Both	7 (28%)	0 (0%)	—

Data are no. (%) of infants unless otherwise indicated.

Abbreviation: IQR, interquartile range

* Includes parainfluenza virus types 1, 2, and 3, influenza virus types A and B, 2009 novel H1N1, human metapneumovirus, coronaviruses NL-65, HKU1, OC43 and 229E, adenovirus, human bocavirus type 1, and enterovirus

FIGURE LEGENDS

Figure 1. Orthogonal partial least squares-discriminatory analysis (OPLS-DA) loadings plot of nasopharyngeal airway metabolomics among infants with bronchiolitis ($n=144$).

This loadings plot combines the covariance (x-axis) and correlation (y-axis) loading profiles resulting from OPLS-DA model. This corresponds to combining the contribution (covariance) with the effect and reliability (correlation) for the model variables (metabolites) with regard to the risk of PPV use. In this figure, we selected and labeled metabolites with higher covariance and correlation that are significantly associated with a higher risk of PPV use (red) or lower risk (blue) in the subsequent analysis. Score scatter plot may be found in supplement (Figure E2).

Abbreviation: PPV, positive pressure ventilation

Figure 2. Support vector machine (SVM) biomarker analysis predicting the risk of positive pressure ventilation use among infants with bronchiolitis ($n=144$). (A) Linear

SVM ROC curves showing increased area under the curve with increased numbers of metabolites. After model 4 (sky blue – 25 metabolites), the prediction was not improved materially, as per Table E2. Model 4 had a sensitivity of 84% and specificity of 86%. (B) Average importance of metabolites from Model 4 (25 metabolites) in predicting the use (or non-use) of PPV based on SVM feature ranking.

Abbreviation: PPV, positive pressure ventilation

Figure 3. Bar plots of the 20 metabolites from the sphingolipid metabolism sub-pathway by use of PPV. Bars represent the average scaled intensity. After adjusting for potential confounders, the intensity of 19 out of 20 metabolites within the sphingolipid metabolism

pathway were significantly different between infants who underwent PPV and those who did not.

* Denotes significance ($P < 0.05$).

Abbreviation: PPV, positive pressure ventilation

Figure 4. Canonical correlation between nasopharyngeal airway microbiota and metabolites associated with positive pressure ventilation use ($n=144$). Relative abundance for the three dominant genera is based on 16S rRNA gene sequencing. Red text indicates metabolites that are associated with a higher risk of PPV use; blue text indicates metabolites that are associated with a lower risk of PPV use. **(A)** Canonical correlation between the relative abundance of dominant bacterial genera and the 25 metabolites from model 4. *Streptococcus* abundance was positively correlated with the intensity of most metabolites from model 4 that are associated with a higher risk of PPV use, such as mannitol-sorbitol, methyl-4-hydroxybenzoate, and 3-(4-hydroxyphenyl)lactate. **(B)** Canonical correlation between the relative abundance of three dominant bacterial genera and the 20 metabolites within the sphingolipid metabolism pathway. *Streptococcus* abundance was positively correlated with the intensity of all 20 metabolites within the pathway.